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Additional antioxidants on the determination of quercetin from *Moringa oleifera* leaves and variation content from different sources

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Abstract

The purpose of this study was to investigate the effect additional synthetic antioxidants have on the determination of quercetin from *Moringa oleifera* leaves and to determine the quercetin content in different sources of *M.oleifera*. Four antioxidants (tert-butylhydroquinone, butylated hydroxytoluene, butylated hydroxyanisole and ascorbic acid) were added in various amounts (2, 5 and 10 mg) to the hydrolysis reaction used to analyze quercetin from crude ethanol extracts of *M.oleifera* leaves. The amount of quercetin recovered from each source was determined by HPLC method, which was appropriate for analysis of quercetin levels in extracts as shown by validation criteria. It was found that addition of either single or mixed antioxidants to the hydrolysis reaction could increase the quantity of quercetin recovered from *M.oleifera* leaves. The amount of quercetin from seven plant materials varied from a low of 3.01 to a high of 13.43 mg/g crude extract. It could be concluded that the analysis of quercetin in *M.oleifera* leaves should add some of the antioxidant in the process of analysis such as BHA 5 mg and TBHQ 5 mg. This could confirm the actual amount found in the leave of this plant and promote the accurate and reliable results to use in the other studies.

Introduction

Moringa oleifera Lam (drumstick tree, or marum in Thai) is the most widely cultivated plant in family Moringaceae. It is one of the multipurpose plants that can be used as vegetable and medicinal plants. At a present time, this plant is already an important crop and is being grown in many countries. All parts of this plant are edible and have long been consumed by humans (Fahey, 2005). It has been used in the traditional medicine in many countries for some symptoms such as skin infections, asthma, bronchitis, cough, sore throat (Ramachandran *et al*., 1980; Fuglie, 2001). Moreover, this plant has been used for nutritional purpose, which comes from various parts enrich of vitamins, minerals and essential amino acid (Babu, 2000). It is especially useful as a food source. Many studies about the pharmacological activities and phytochemical constituents of this plant were also reported (Caceres *et al*., 1991; Bharali *et al*., 2003; Sreelatha and Padma, 2009). Some active constituents found in this plant have been reported to have antioxidant, hypotensive, spasmolytic, antifungal, anticancer, antibacterial activities and hypocholesterolemic effects (Caceres *et al*., 1992; Metha *et al*., 2003; Chuang *et al*., 2007).

This plant was reported to contain benzyl glucosinolates, ß-sitosterol, glycosides, sugars, alkaloids, flavonoids, proteins and saponins (Goyal *et al*., 2007). Quercetin belongs to an extensive class of polyphenolic flavonoid compounds and has some biological activities such as antibacterial, antioxidant and anticarcinogenic activities (Williamson *et al*., 1996; Lamson, 2000; Rahman *et al*., 2009). Quercetin usually combined with a sugar moiety and was found as the major constituent in the leaves of this plant (Lago *et al*., 2007). However, the analytical method to determine flavonoid glycoside is quite difficult. Hydrolysis reaction need to used and the resulting quercetin can be identified and quantified. In order to determine the amount of quercetin in the leave of this plant without degrading the aglycone itself during the acid hydrolysis in sample preparation procedures, the effect of addition some antioxidant was tested in this study. During the hydrolysis reaction which quercetin glycoside changed to quercetin, we believed that if

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antioxidant was not added into the reaction, it could cause degradation of quercetin and the content would be less than it should be. One of the most common analytical methods is High performance liquid chromatography, which can be used to quantify the major flavonol components in some medicinal plants (Dubber and Kanfer, 2004). The modified HPLC method with selective and accurate results is necessary to support this purpose (Hertog *et al*., 1992; Nuutila *et al*., 2002). The information of active constituents is used in order to ensure the reliability and consistency of using this plant. Thus, the present research aimed to study the effect of some additional antioxidant to the analysis of quercetin in *M.oleifera* leaves. We therefore focused our study on the determination of quercetin content from varying sources using the optimize amount antioxidants addition resulted by before study.

Materials and Methods

Plant materials and reagents

M.oleifera leaves in different age groups (No.1 > 5 years, No.2-6 ~ 2-4 years, No.7 < 2 years) were collected from Khon Kaen province, Thailand and their identity was confirmed by Dr. Prathan Luecha, Faculty of Pharmaceutical Sciences, Khon Kaen University. A voucher specimen (SD 5501) has been deposited in the Herbarium of the Faculty of Pharmaceutical Sciences, Khon Kaen University. Chemicals used in this study were obtained from the following sources: tert-butylhydroquinone (TBHQ), butylated hydroxyl toluene (BHT), butylated hydroxyanisole (BHA), ascorbic acid (ASA) and formic acid from Fluka®, methanol and hydrochloric acid from BDH®, 2-propanol, acetonitrile and ethanol from Merck®, standard quercetin from Sigma Aldrich®.

Preparation of the extracts

The leaves were dried at 50°C, powdered and macerated with ethanol for 7 days. Ethanol was the best choice in this study because this ethanol extract would be used for herbal formulation and safe to add into the preparation. The extracts were then filtered and concentrated to dryness by a rotary evaporator.

Hydrolysis of crude extracts

The hydrolysis method (Hertog, 1992) was used in this study. *M. oleifera* leaf extracts (50 mg) were refluxed at 80°C for 2 h with 1.2 M hydrochloric acid in 50% aqueous methanol (5 ml). Antioxidants (TBHQ, BHA, BHT and ASA) were added to the hydrolysis reaction in various amounts (2, 5 and 10 mg) before refluxing. After refluxing, each sample was allowed to cool to room temperature and the volume was adjusted to 10 ml with methanol. All determinations were performed in triplicate.

HPLC analysis of quercetin

The hydrolyzed samples were analyzed by reversed-phase HPLC system using a Hypersil ODS column (Agilent®, 4x250 mm, 5 µm). An acetonitrile: 2-propanol: 1% formic acid solution (8:22:70) was used as the mobile phase at the flow rate of 1 ml/min and quercetin was detected at 370 nm. The amount of quercetin present in hydrolyzed extracts was then determined from comparison with a quercetin standard curve (0.48 µg/ml, 0.96 µg/ml, 1.92 µg/ml, 4.8 µg/ml, and 9.6 µg/ml).

Validation of analytical method

The analytical method was validated for specificity by the absence of undesired peaks in HPLC chromatograms and accuracy by the percentage recovery of quercetin standards in 5 replicates. Precision (%RSD) was validated for within-day and between-day determinations (n = 5) and for HPLC retention times (n = 10). Linearity was validated by using linear regression analysis to calculate the coefficient of determination (r^2) of a quercetin standard curve (0.48 µg/ml, 0.96 µg/ml, 1.92 µg/ml, 4.8 µg/ml, and 9.6 µg/ml, n = 5). Limit of detection (LOD) and limit of quantitation (LOQ) were determined by using diluted standard quercetin solutions and a signal to noise ratio of 3.42 and 10 respectively (n = 5). Finally, the reproducibility and consistency of the hydrolysis procedure was validated by determining the %RSD from ten replicate hydrolysis procedures.

Statistical analysis

The data were expressed as means ± SD. Results were compared by two-way ANOVA. Differences were considered significant if $p < 0.05$.

Variation of quercetin content from seven sources of M.oleifera leave extracts

Seven plant materials of M. oleaifera leave were collected from different area of Khon kaen province. The extracts of these plant materials were prepared as the extraction method used before. The amount of quercetin content in each sample was also determined by the HPLC method and the hydrolysis reaction was also used as described above. The optimized amount of antioxidant received from this study (TBHQ 5 mg and BHA 5 mg) was added prior to the analysis. The analysis was performed in three replications.

Results and Discussion

Analytical method validation

HPLC chromatograms showed good separation of peaks, with resolution (R_s) values more than 2 and tailing factors (T) less than 2 (Figure 1). The within-day and between-day percentage relative standard deviations (%RSD) for the five replicate injections of 0.48 µg/ml, 0.96 µg/ml,1.92 µg/ml, 4.80 µg/ml and 9.60 µg/ml quercetin standards were all less than 1% (Table 1). The calibration curve obtained from plotting the peak area at each concentration had good linearity (correlation coefficient $r^2 = 0.9998$, Figure 1). Accuracy, as determined by the percentage recovery of spiked quercetin standards, was always between 90-110% (Table 1). Finally, the reproducibility of the hydrolysis procedure was assessed by carrying out ten replications with the same extract. The %RSD of quercetin content was 3.65 (Table 1). The remaining validation data obtained for this HPLC method (Retention time, LOQ and LOD) are reported in Table 1. According to ICH guideline, all the validation criteria were in acceptance range and could be used for quercetin analysis in the extract

Table 1. Validation results of the HPLC analytical method for quercetin determination

Validation criteria	N	Factor	Result
Accuracy	5	% Recovery	91.66% - 102.81%
Within Day Precision	5	0.48 µg/mL	0.44% RSD
		0.96 µg/mL	0.23% RSD
		1.92 µg/mL	0.13% RSD
		4.80 µg/mL	0.08% RSD
		9.60 µg/mL	0.03% RSD
Between Day Precision	5	0.48 µg/mL	0.57% RSD
		0.96 µg/mL	0.15% RSD
		1.92 µg/mL	0.09% RSD
		4.80 µg/mL	0.03% RSD
		9.60 µg/mL	0.03% RSD
Retention Time	10		0.27% RSD
Linearity	5	r^2	0.9998 (± 0.0000)
LOD	5	S/N = 3.42	0.16 µg/mL
LOQ	5	S/N = 10.00	0.48 µg/mL
Hydrolysis Procedure	10		3.65% RSD

The effect of antioxidant type and concentration on the recovery of quercetin from M. oleifera leaf extracts.

To determine the effect of additional antioxidants to the hydrolysis mixture, various amounts (0, 2, 5 and 10 mg) of TBHQ, BHA, BHT and ASA were added into the hydrolysis reaction and the amount of quercetin aglycone recovered was determined. Table

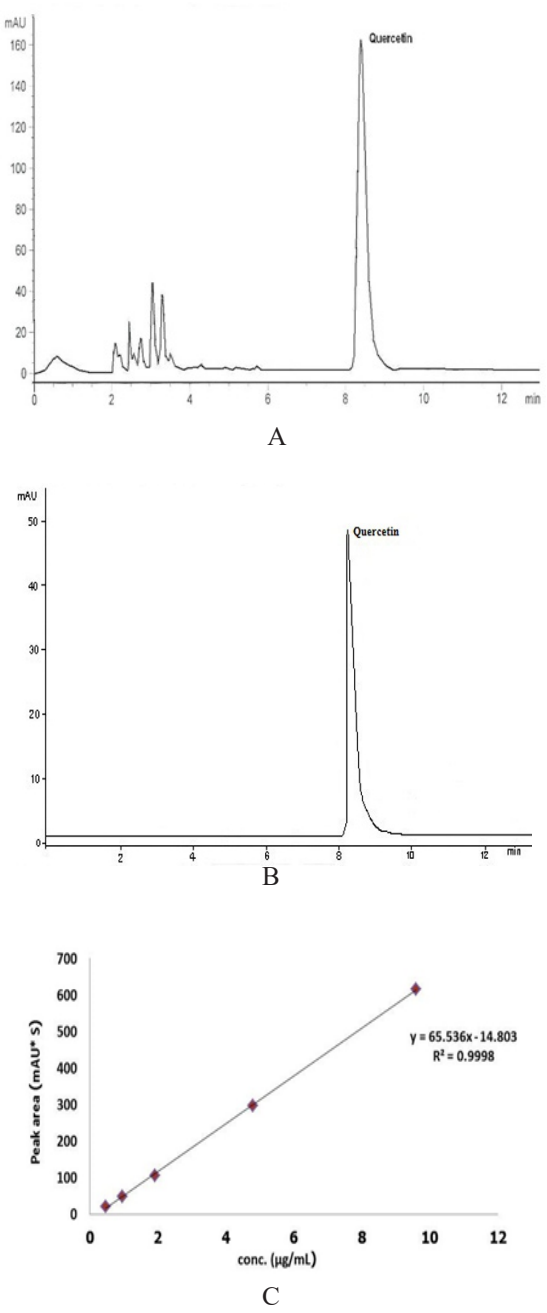

Figure 1. HPLC chromatograms of *M. oleifera* leaf extract (A), a standard quercetin solution (B) and calibration curve of standard quercetin solution (C)

2 showed that the amount of quercetin recovered varied for each antioxidant and each concentration. Ten mg of TBHQ and ten mg of BHA yielded the most quercetin per gram of extract: 11.25 (±1.58) and 11.25 (±1.34), respectively (Table 2) From the statistical analysis, only BHT was found different to the other antioxidants (TBHQ, BHA and ASA). Addition of mixtures of antioxidants to the hydrolysis reaction gave increased yields of quercetin compared to individual antioxidants, as shown in Table 3. The tend highest yield of quercetin was 13.43 (±0.43) mg/g extract with the mixture of 5 mg TBHQ and 5 mg BHA.

Table 2. The effect of individual antioxidants on the recovery of quercetin from *M. oleifera* leaves (n = 3)

Type of antioxidant	mg of antioxidant	Quercetin content mg/g extract ± SD
NONE	0	10.15 ± 0.66
TBHQ	2	10.90 ± 1.40
	5	10.95 ± 1.52
	10	11.25 ± 1.58
BHT	2	8.31 ± 1.18
	5	9.15 ± 2.06
	10	9.17 ± 2.34
BHA	2	11.09 ± 2.36
	5	11.23 ± 0.57
	10	11.25 ± 1.34
ASA	2	10.63 ± 0.48
	5	10.76 ± 0.63
	10	10.97 ± 1.19

TBHQ; tert-butylhydroquinone, BHT; butylated hydroxyl toluene, BHA; butylated hydroxyanisole, ASA; ascorbic acid

Table 3. The effect of antioxidant mixtures on the recovery of quercetin from *M. oleifera* leaves (n = 3)

Amount of antioxidant (mg)			Quercetin content mg/g extract ± SD
TBHQ	BHA	ASA	
2	5	0	12.47 ± 0.45
5	5	0	13.43 ± 0.43
2	0	2	13.36 ± 1.34
5	0	2	12.96 ± 0.98
2	0	5	12.01 ± 0.97
5	0	5	12.35 ± 3.11
0	5	5	11.70 ± 3.29
0	5	2	10.80 ± 2.70

TBHQ; tert-butylhydroquinone, BHA; butylated hydroxyanisole, ASA; ascorbic acid

The addition of single antioxidants to the hydrolysis mixture prior to the analysis of quercetin generally resulted in higher amounts of quercetin recovered. This confirms the study of Nuutila from 2001 in which the quercetin content in red spring onion was found to be higher after addition of TBHQ or ASA to the hydrolysis reaction. In our study, tendency of higher quercetin content was found with the addition of TBHQ, BHA and ASA to the hydrolysis reaction, and quercetin content was lower with the addition of BHT, compared to no antioxidant controls. This suggests that BHT somehow protected quercetin during the hydrolysis process, blocking the release of the quercetin aglycone. For the mixed antioxidants, the addition of TBHQ (5 mg) and BHA (5 mg) to the hydrolysis reaction tend resulted in the highest amount of quercetin recovered. Thus, by using an antioxidant mixture, lower total amounts of antioxidants could be added to the hydrolysis reaction, conceivably reducing quercetin aglycone decomposition. The accurate result of quercetin content in *M.oleifera* could be obtained from the optimized additional antioxidants which performed the advantage of this study. However, this condition was optimized only for one kind of plant, *M.oleifera*, if the study conducted to the other plants, the other optimized condition should be s*et al*so.

Variation of quercetin content from different sources

From the varying quercetin content from various sources in *M. oleifera* extract. It was notice that the content varied from 3.01 to 13.43 mg/g extract, which showed very wide range (Table 4).

From the information of these plant materials with biodiversity, we did not grow and control the condition of this plant. These plant materials were provided from many sources and different ages of these samples were collected. The highest amount of quercetin content was recovered from the plant samples with the highest age which much more than 5 years. Thus, the study about biodiversity of plant sources should be performed to obtain the information that could help the managing of the time to collect the plant material which could give the high content of quercetin. This could help for the plant selection process.

Table 4. Amount of quercetin recovered from various sources of *M. oleifera* leaves. (n = 3)

Source Number	Quercetin content mg/g extract ± SD
1	13.43 ± 0.43
2	7.94 ± 1.01
3	8.88 ± 1.50
4	8.65 ± 0.75
5	7.05 ± 0.70
6	5.99 ± 0.16
7	3.01 ± 0.69

Conclusion

From the results of this study, we suggested that the analysis of quercetin in *M. oleifera* leaves was improved by the addition of an antioxidant such as tert-butylhydroquinone (TBHQ), butylated hydroxyanisole (BHA) or a mixture of antioxidants (TBHQ + BHA) during the hydrolysis process. The selection of plant material to obtain the high content of active ingredient could be done if we knew the collecting condition. Here we reported an optimized method for quercetin analysis in *M. oleifera* leaf extracts, and the procedure could be used to determine the content of other flavonoid glycosides from plants or natural sources.

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